An Investigation of the Ability of Antipsoriatic Drugs to Inhibit Calmodulin Activity: A Possible Mode of Action of Dithranol (Anthralin)*

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Epidermal calmodulin (CaM) has been reported to be elevated in psoriasis and to decrease following clearance of psoriasis with treatment. We set out to investigate whether any of the principle drugs used in the treatment of psoriasis had inherent CaM antagonist activity. Utilizing a CaM-activated phosphodiesterase we have demonstrated that even at very high concentrations, the systemic drugs etretinate, methotrexate, and 8-methoxypsoralen, and the topical agents hydrocortisone and crude coal tar showed minimal CaM inhibitory activity. Dithranol (anthralin), however, whether freshly prepared or oxidized, produced substantial inhibition of CaM activity and was demonstrated to be a potent competitive antagonist of CaM, suggesting another possible therapeutic mode of action of dithranol in psoriasis. *J Invest Dermatol 87:232–235, 1986*

Levels of the intracellular calcium binding protein calmodulin (CaM) have been shown to be elevated in both lesional [1–5] and nonlesional [2–5] psoriatic epidermis. CaM regulates a wide range of intracellular processes, several of which have been reported to be altered in psoriasis; thus CaM activates a number of enzymes including cyclic nucleotide phosphodiesterase, phospholipase A2, and ornithine decarboxylase, whose levels and those of their products are known to be abnormal in psoriasis [6–8]. In addition, the epidermis is hyperproliferative in psoriasis, and there is a considerable body of evidence that CaM is essential for the regulation of cell division [9]. An alteration in the regulation of CaM activity in the psoriatic epidermis would therefore help to explain a number of seemingly unrelated abnormalities.

We have recently shown that a successful response to treatment in psoriasis, by a variety of established treatment regimens, is associated with a decrease in epidermal CaM activity to within the normal range for control volunteers with little change in those individuals where CaM levels were already low [10]. This latter study led us to consider whether any of the established topical and systemic methods of treatment for psoriasis (for some of which the mode of action in psoriasis is not clearly established) had any inherent CaM antagonist activity. Accordingly we have investigated the ability of 3 topical agents, dithranol, hydrocortisone, and crude coal tar, and 3 systemic drugs, etretinate, methotrexate, and 8-methoxypsoralen to inhibit the CaM activation of a CaM-sensitive enzyme, beef heart phosphodiesterase.

MATERIALS AND METHODS

Assessment of the Effect of Drugs on Calmodulin Activity Calmodulin activity was measured based on its activation of a CaM-dependent beef heart phosphodiesterase (Boehringer Mannheim, London) as described previously [11]. Assays contained in a final reaction mixture of 400 μl, 40 mM Tris-HCl, pH 7.0 at 37°C, 4 mM 2-mercaptoethanol, 5 mM $MgCl_2$, ^3H-labeled cyclic AMP (2×10^5 cpm/tube) (Amersham International Limited, Bucks, U.K.), 100 μM cyclic AMP (Boehringer Mannheim, London), 25 μM $CaCl_2$, and pure CaM and drugs as required. Pure CaM was prepared from pig brain by the method of Kakiuchi et al [12].

Incubations were for 15 min at 37°C. For assessment of the potential inhibitory effects of the drugs, assays contained sufficient CaM (12.5 ng/400 μl incubation) to produce 60–80% of the maximum response to CaM. The antipsoriatic drugs tested were etretinate (Roche Products Limited, Welwyn Garden City, Herts, U.K.), methotrexate (Lederle Laboratories Division, Cyanamid of Great Britain Ltd.), 8-methoxypsoralen (supplied as a gift from Upjohn Ltd., Fleming Way, Crawley, West Sussex), crude coal tar (Thornton & Ross, Linthwaite Laboratories, Huddersfield), dithranol (Staveley Chemicals Ltd., Staveley Works, Chesterfield, Derbyshire), and hydrocortisone acetate (The Boots Company PLC, Nottingham). Two oxidative products of dithranol, 1,8-dihydroxyanthraquinone and dithranol dehydrodimer, were also kindly supplied by Dr. M. Whitefield of Dermal Laboratories Ltd., Hitchin, Hertfordshire. The CaM antagonist N-(6-aminohexyl)-5-chloro-1-naphthalene sulphonamide (W7) (supplied as a gift from Dr. M. Blackburn, Department of Chemistry, Sheffield University) was also used for comparative purposes in this study. For etretinate and 8-methoxypsoralen it was necessary to solubilize these drugs first in DMSO, and 2% DMSO was accordingly added to all incubations with these drugs. Crude coal tar and dithranol were solubilized first in ethanol and 2.5% ethanol was present throughout all incubations with these drugs. (DMSO at 2% and ethanol at 2.5% significantly lowered both CaM-inde-

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Abbreviations:

CaM: calmodulin

W7: N-(6-aminohexyl)-5-chloro-1-naphthalene sulfonamide

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pendent and CaM-dependent phosphodiesterase activity. The response of the enzyme to CaM was not itself affected.)

Each drug was tested over a wide range of concentrations (all were tested up to 2.5×10^{-3} g/liter and higher where solubility permitted) in order to include those concentrations likely to be achieved systemically or locally in clinical usage.

Incubations were performed in triplicate and each experiment was repeated on a minimum of 3 occasions.

Student's t-test was used to evaluate the statistical significance of the effects of the drugs on CaM activity.

RESULTS

The majority of the antipsoriatic drugs studied showed minimal CaM inhibitory activity even at very high concentrations (see Fig 1). Thus, methotrexate at 2.5×10^{-3} g/liter, etretinate at 2.5×10^{-3} g/liter, 8-methoxypsoralen at 2.5×10^{-1} g/liter, hydrocortisone at 2.5×10^{-1} g/liter, and crude coal tar at 2.5×10^{-2} g/liter inhibited CaM-dependent activation of beef heart phosphodiesterase by 20% or less. (Although 8-methoxypsoralen had no CaM antagonist properties in its own right, we should point out that it is normally given clinically in combination with UVA irradiation). Solubility problems with etretinate and coal tar prevented testing of these substances at any higher concentrations.

The degree of inhibition achieved at these concentrations varied from experiment to experiment and, overall, did not achieve statistical significance ($p > 0.05$). Dithranol, however, significantly inhibited CaM activity (Fig 1) with 50% inhibition of CaM-dependent phosphodiesterase activity occurring at $5.5 \pm 0.04 \times 10^{-3}$ g/liter ($\pm$SEM, n = 3 experiments), equivalent to 24 μM ($p < 0.005$). In comparison, in these experiments the CaM antagonist W7 produced 50% inhibition (IC_{50}) at 1×10^{-2} g/liter, equivalent to 29 μM.

We examined the inhibitory effect of dithranol (1,8-dihydroxy-9-anthrone) in greater detail as shown in Figs 2 and 3. Dithranol exposed to air and sunlight quite quickly becomes discolored as it is oxidized to danthron (1,8-dihydroxyanthraquinone) and dianthrone, a dithranol dehydrodimer (1,8,1′,8′-tetrahydroxy-10,10′-bi-9[10H]-anthrone). We examined the CaM antagonist properties of freshly prepared dithranol and of oxidized dithranol solutions and found both inhibited CaM activity to a similar extent, with the sole difference being that fresh dithranol also inhibited basal phosphodiesterase activity which is CaM-independent (Fig 2). Oxidized dithranol solutions did not affect basal enzyme activity even up to 2.5×10^{-2} g/liter.

We confirmed the CaM-antagonist properties of oxidized dithranol solutions by examining pure preparations of the 2 most common oxidation products of dithranol—danthron and the dithranol dehydrodimer. The former was equipotent with dithranol in inhibiting CaM activity; the latter appeared to be slightly less

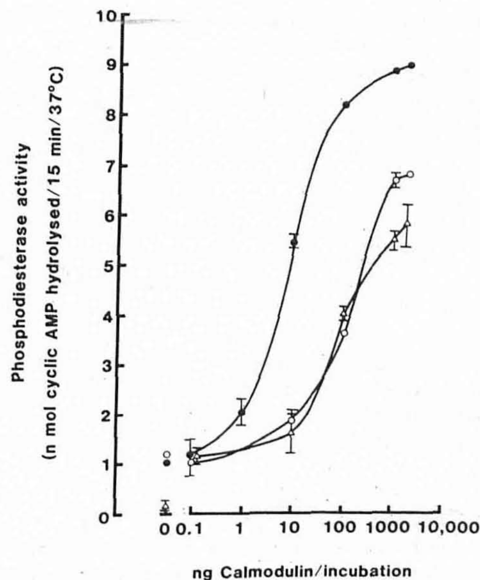

Figure 2. The effect of dithranol on the ability of CaM to stimulate beef heart CaM-dependent phosphodiesterase activity. Incubations contained CaM alone (*closed circle*) or CaM plus 2.5×10^{-2} g/liter freshly made up dithranol (*open triangle*) or CaM plus 2.5×10^{-2} g/liter dithranol allowed to stand in the sun for approximately 3 weeks (*open circle*).

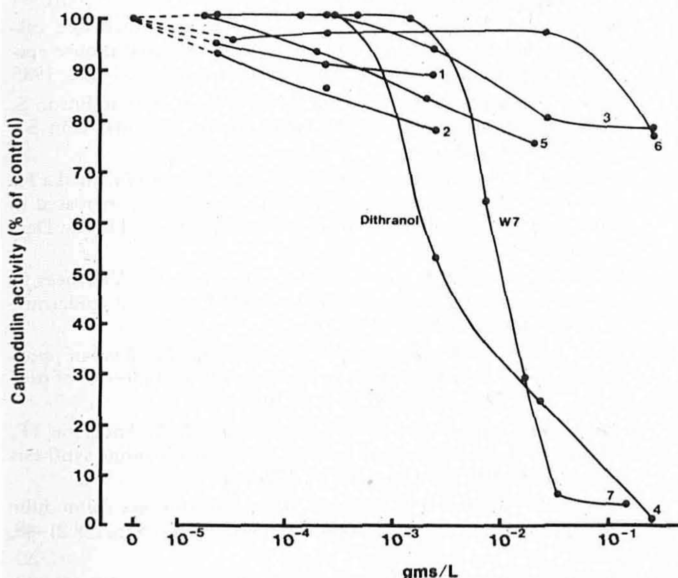

Figure 1. The effects of methotrexate (*1*), etretinate (*2*), 8-methoxypsoralen (*3*), dithranol (*4*), crude coal tar (*5*), hydrocortisone (*6*), and the CaM antagonist N-(6-aminohexyl)-5-chloro-1-naphthalene sulfonamide (W7) (*7*) on CaM-activated phosphodiesterase. The results shown are of single representative experiments for each drug (repeated on at least 2 other occasions). Data from separate experiments are combined by designating the increase in phosphodiesterase activity due to CaM (CaM-independent enzyme activity of approximately 20% is subtracted) as 100%.

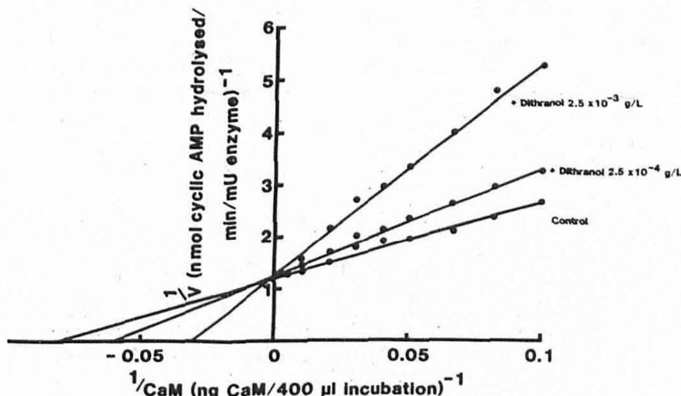

Figure 3. Kinetic analysis of the dithranol-induced inhibition of activation of beef heart phosphodiesterase by pure CaM. Phosphodiesterase activity was measured in the presence of 0, 2.5×10^{-4} g/liter, and 2.5×10^{-3} g/liter dithranol over a range of CaM concentrations. Data are plotted as 1/V vs 1/CaM, where V is nmol cyclic AMP hydrolyzed min^{-1} mU phosphodiesterase and CaM concentration is ng of CaM per 400 μl incubation. Each *point* represents the mean of triplicate determinations. The increase in slope due to 2.5×10^{-3} g/liter dithranol was used to calculate K_i using the relationship that this increase represents $1 + [I]/K_i$.

potent, although the extremely low water solubility of the dehydrodimer made working with this form extremely difficult.

Increasing concentrations of CaM reduced the inhibition produced by dithranol whereas increasing concentrations of calcium (up to 1 mM) (on which the enzyme is also dependent) did not affect the inhibition. Kinetic analysis of the inhibition using a double-reciprocal Lineweaver-Burk plot (Fig 3) showed that the V_{max} (y axis intercept) values for the CaM-stimulated enzyme activity in the absence of drug and in the presence of 2.5×10^{-4} and 2.5×10^{-3} g/liter dithranol were the same and that the apparent K_m (slope $= K_m/V_{max}$) of CaM decreased with increasing concentrations of dithranol. This is consistent with dithranol acting as a competitive inhibitor of CaM. The increase in slope in the double-reciprocal plot produced by 2.5×10^{-3} g/liter dithranol was used to calculate the inhibitor constant K_i [13], using the relationship that this increase in slope is equivalent to $1 + [I]/K_i$ [10]. This gave an inhibitor constant for dithranol of 2.5×10^{-3} g/liter.

DISCUSSION

We have previously reported that both biologically active and radioimmunoassayable levels of CaM are increased 2- to 3-fold in the psoriatic plaque, and are also slightly increased in the uninvolved epidermis in psoriasis [2,4]. Our finding that there is an intrinsic elevation in the nonlesional epidermis has now been confirmed by 2 other groups, Fairley et al [3] and Mizumoto et al [5]. The 2- to 3-fold elevation in CaM seen by these workers and by us in the psoriatic plaque is of a similar order to that reported in virally transformed cells [14], and to the CaM levels we have observed in various neoplastic tissues. Cellular hyperproliferation alone, however, does not seem to explain this elevation, since we have found no significant increase in CaM levels in normal control epidermis induced to proliferate by plastic tape stripping, or in lymphocytes stimulated to divide by phytohemagglutinin [4].

Whether or not increased CaM activity in psoriatic epidermis is one of the initiating biochemical changes in psoriasis, we have shown that clinical improvement in psoriasis is associated with decreased CaM activity in the lesional epidermis [10] (CaM activity in the uninvolved epidermis remained slightly elevated). The possibility that some of the current psoriasis treatment regimens might affect CaM activity directly seemed worthy of examination, particularly as a wide variety of structurally dissimilar drugs can inhibit CaM.

Our results show that dithranol alone, out of the 6 drugs tested, had any direct CaM antagonist activity as determined by the ability of these drugs to inhibit CaM-dependent beef heart phosphodiesterase activity. Dithranol has been shown in this study to be a potent competitive inhibitor of CaM at concentrations within the range likely to be encountered in clinical usage. The mechanism of dithranol's therapeutic action in psoriasis has remained obscure for many years. Dithranol has been reported to inhibit glucose-6-phosphate dehydrogenase [15] and several glycolytic enzymes in vitro [16], to reduce mitotic activity and DNA synthesis [17–20], and to act as an uncoupler of oxidative phosphorylation in mitochrondria [21]. The ability to inhibit CaM would seem to be equally important and may also explain the effects of dithranol on DNA synthesis which is known to be inhibited by CaM antagonists [22]. However, oxidized forms of dithranol are reported to be less effective therapeutically [19,23] and we find fresh and oxidized forms to be equipotent in inhibiting CaM activity. One is led to the conclusion that either the CaM antagonist activity of the drugs is irrelevant to their action in psoriasis or that oxidized forms of dithranol should also be effective clinically. It has been pointed out that the inherent instability of dithranol which leads in vitro and possibly in vivo to the formation of other derivatives makes it difficult to determine which is the active species in the skin [24]. Not all of the biologic actions of dithranol and its oxidative products can be accommodated in a consistent coherent picture of how dithranol acts in psoriasis—for example, the species of dithranol and its products which have been found to be active against glucose-6-phosphate dehydrogenase [25] are different from those which inhibit DNA synthesis and induce cytotoxicity [26,27].

It has recently been reported that mitochondrial function is the most sensitive target in the cell for dithranol activity (an effect mimicked to a lesser extent by the dithranol dimer but not by danthron), suggesting that this may be dithranol's mode of action clinically [28]. However, dithranol has also been reported to inhibit DNA repair [29] (danthron much less so) and very recently a role for CaM in DNA repair has also been proposed [30]. Accordingly, we would argue against premature discarding of any new information about the biologic properties of dithranol and its oxidative products.

Several drugs whose clinical modes of action are not fully established have recently been found to possess CaM antagonist properties (for example Tamoxifen [31]), which may contribute to their therapeutic actions. Caution must be exercised, however, since despite their CaM antagonist activity, some drugs such as propranolol, in practice, exacerbate psoriasis. In the case of propranolol, the explanation may be that inhibition of adenylate cyclase by this drug, with a consequent fall in intracellular cyclic AMP levels, takes precedence over a weak CaM inhibition [32]. No reduction in disease severity is seen among coincidentally psychotic psoriatic patients treated with large doses of oral or parenteral phenothiazines. Hence, it is possible that, in order to have an antipsoriatic effect, a sufficient concentration of a CaM antagonist can be achieved safely only by topical application.

The discovery that dithranol, an established topical treatment for psoriasis, possesses CaM antagonist properties which may at least partially explain its therapeutic efficacy, nevertheless provides a stimulus to investigate the use of less toxic CaM antagonists in the management of this disease.

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